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RESEARCH ARTICLE

In vitro inhibition of polyphenol oxidase by some new diarylureas

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Abstract

A new series of N,N′-diarylureas **(1–9)** was synthesized. These compounds were investigated as inhibitors of polyphenol oxidase (PPO) which had been purified from banana by an affinity gel comprised of Sepharose 4B-L-tyrosine-*p*-amino benzoic acid. K_i values for **(1), (2), (3), (5), (6), (7)** and **(8)** were determined as 0.285, 17.97, 0.187, 0.108, 0.063, 0.044 and 0.047 mM, respectively. Thus **(2)** was by far the most effective inhibitor. Interestingly, **(4)** and **(9)** behaved as an activator of PPO in this study.

Keywords: Inhibition, enzymatic browning, polyphenoloxidase, diarylureas

Introduction

Polyphenol oxidase (PPO) (EC 1.14.18.1) is a copper-containing enzyme, widely distributed in nature, responsible for melanization in animals and browning in plants[1,2]. PPO also catalyzes the *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones[1]. Enzymatic browning of fruits is related to oxidation of phenolic endogenous compounds into highly unstable quinones, which are later polymerized to brown, red and black pigments[3]. The degree of browning depends on the nature and amount of endogenous phenolic compounds, presence of oxygen, reducing substances and metallic ions, pH and temperature and the activity of PPO, the main enzyme involved in the reaction[4]. Enzymatic browning is also an economic problem for processors and consumers[1,5]. At least five causes of browning in processed and/or stored fruits and plants are known: enzymatic browning of the phenols, Maillard reaction, ascorbic acid oxidation, caramelization and formation of browned polymers by oxidized lipids[6].

Browning reactions are major causes of quality loss during harvesting, post-harvest handling/storage and processing of fruits, plants and vegetables in food industry[7]. The enzymatic browning cause deterioration of sensory and nutritional quality and affects appearance and organoleptic properties, inactivation of PPO is desirable for preservation of foods[8]. Several methods such as the addition of antioxidants and the exclusion of oxygen as well as thermal processing have been used to inhibit enzymatic browning. For inactivation of PPO, thermal processing has limits like loss of sensory and nutritional quality of food products. Therefore, high pressure treatment has been considered as an alternative[9,10].

Ureas are very important class of carbonyl compounds. They have extensive applications such as agrochemicals, dyes for cellulose fibers, antioxidants in gasoline, resin precursors and synthetic intermediates for fine chemicals, pharmaceuticals, cosmetics and pesticides[11–16]. N,N′-diarylureas are valuable starting material used in organic synthesis. They have numerous applications such as drugs, pesticides, herbicides, antioxidants and anion-binding receptors[17]. Many urea derivatives are very important compounds because of their biological activities. In particular, several substituted ureas have recently shown an inhibiting effect on HIV protease enzyme[11–13].

In the present study, we have synthesized derivatives of N,N′-diarylureas for evaluation as potential inhibitors

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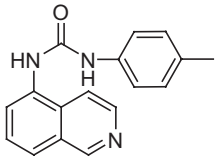
of PPO that could be beneficial in the prevention of enzymatic browning.

Materials and methods

General procedure for the synthesis of N,N′-diarylureas

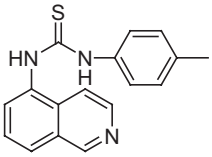
Chemicals and solvents used in the study were obtained from Sigma-Aldrich and Merck and were used without further purification. Melting points were measured on Barnstead/Electrothermal 9200 melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury NMR at 300 and 75 MHz instrument in $CDCl_3$, respectively. IR spectra were obtained with Shimadzu IR Prestige 21.

1



1-(isoquinolin-5-yl)-3-*p*-tolylurea (**1**): 1-isocyanato-4-methylbenzene (6.94 mmol, 0.924 g) was dissolved in toluene (10 ml) and added dropwise into the stirred solution of 5-aminoisoquinoline (6.94 mmol, 1 g) in tetrahydrofuran (15 ml). The reaction mixture was stirred at 60°C overnight. The precipitate was collected by filtration and washed with acetone (5 ml) and dried under vacuum at 40°C. Yield 95%; mp: 251–252°C; ^{1}H NMR (DMSO-d_6): δ 2.2 (s, 3H), 7.0–7.1 (d, 2H), 7.36–7.39 (d, 2H), 7.56–7.59 (dd, 1H), 7.69–7.7 (d, 1H), 7.7–7.71 (d, 1H), 8.0–8.06 (d, 1H), 8.49–8.52 (d, 1H), 8.89 (d, 1H), 8.9 (s, 1H), 8.9 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 21.05, 117.99, 118.97 (2C), 121.40, 121.74, 124.40, 129.96 (2C), 130.12, 130.85, 131.51, 135.54, 137.70, 148.86, 150.98, 153.55; FT-IR ν (cm^{-1}): 3140, 2966, 1595, 1541, 1498, 1444, 1332, 1263; (MH$^+$): 276.1.

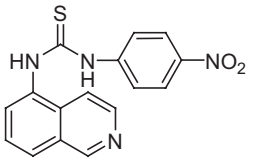
2



1-(isoquinolin-5-yl)-3-*p*-tolylthiourea (**2**): The experimental procedure is the same as described above. Yield 90%; mp: 184–185°C; ^{1}H NMR (DMSO-d_6): δ 3.26 (s, 3H), 7.11–7.14 (d, 2H), 7.33–7.36 (d, 2H), 7.54–7.56 (d, 1H), 7.55–7.58 (d, 1H), 7.71–7.76 (t, 1H), 7.91–7.94 (d, 1H), 8.31–8.34 (d, 1H), 8.8 (s, 1H), 9.7 (s, 1H), 9.8 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 21.23, 121.99, 125.05 (2C), 126.05, 126.16, 128.29, 129.62 (2C), 129.72, 132.67, 134.68, 136.37, 137.45, 148.99, 151.17, 182.14; FT-IR ν (cm^{-1}): 3134, 2972, 1595, 1541, 1498, 1444, 1332, 1263; MS(MH$^+$): 293.1.

General reaction

3



	X	R_1	R_2	R_3	R_4	R_5
1	O	-H	-H	$-CH_3$	-H	-H
2	S	-H	-H	$-CH_3$	-H	-H
3	S	-H	-H	$-NO_2$	-H	-H
4	O	-H	-H	-F	-H	-H
5	S	-H	-I	-H	-H	-H
6	S	-H	-F	-H	-H	-H
7	S	-F	-H	-H	-H	-H
8	S	-H	$-CF_3$	-H	-H	-H
9	S	-H	-Cl	-H	-Cl	-H

Purification of PPO

All purification steps were carried out at 25°C. The extraction procedure was adopted from Wesche-Ebeling and Montgomery[18]. The bananas were washed with distilled water three times to prepare the crude extract, 50 g of bananas were cut quickly into thin slices and homogenized in a Waring blender for 2 min using 100 ml of 0.1 M phosphate buffer, pH 7.3 containing 5% poly(ethylene glycol) and 10 mM ascorbic acid. After filtration of the homogenate through muslin, the filtrate was centrifuged at 15,000*g* for 30 min, and the supernatant was collected. A crude protein precipitate was made by adding $(NH_4)_2SO_4$ to 80% saturation. The resulting precipitate was suspended in a minimum volume of 5 mM phosphate buffer and then dialyzed against 5 the same buffer overnight. The enzyme solution was then applied to the Sepharose 4B-tyrosine-*p*-amino benzoic acid affinity column[7], pre-equilibrated with 5 mM phosphate buffer, pH 5.0. The affinity gel was extensively washed with the same buffer before the banana PPO (BPPO) was eluted with 1 M NaCl, 5 mM phosphate, pH 7.0.

BPPO activity

Enzyme activity was determined using catechol by measuring the increase in absorbance at 420 nm[19] in a Biotek automated recording spectrophotometer. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 unit/min for 1 ml of enzyme at 25°C[7].

Inhibition of BPPO activity

An aliquot of each inhibitor at various final concentrations was added to the standard reaction solution immediately before the addition of enzyme extract.

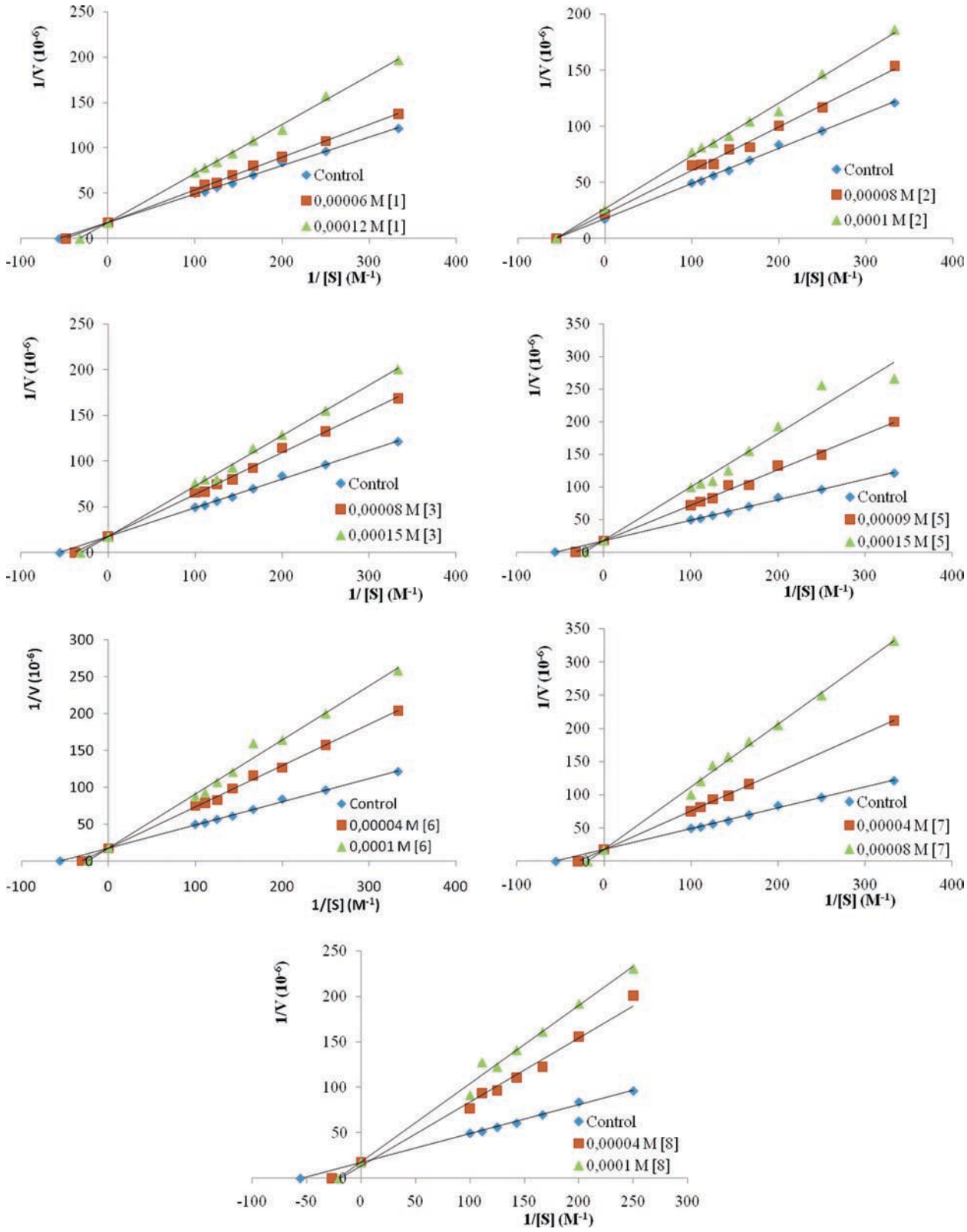


Figure 1. K_i graphics of diarylureas on BPPO.

The concentration of inhibitor (diarylureas) giving 50% inhibition was determined from a plot of residual activity against inhibitor concentration, with 10 mM catechol as substrate. The control was activity without inhibitor.

Results and discussion

The inhibition type of diarylureas was determined by Lineweaver–Burk plots of 1/V versus 1/S at two inhibitor concentrations (Figure 1). The inhibition constant, K_i,

Table 1. The K_i values of diarylureas on BPPO.

Diarylureas	K_i (mM)	Inhibition type
1	0.285	Competitive
2	17.97	Non-competitive
3	0.187	Competitive
4	–	–
5	0.108	Competitive
6	0.063	Competitive
7	0.044	Competitive
8	0.047	Competitive
9	–	–

was deduced from the points of interception of the plots. Depending on kinetic analysis, competitive inhibition **(1, 3, 5, 6, 7, 8)** and uncompetitive inhibition **(2)** were all seen in this study (Table 1). Surprisingly, neither **(4)** nor **(9)** had much of an inhibiting effect, in any of the conditions used. K_i values of 0.285, 17.97, 0.187, 0.108, 0.063, 0.044 and 0.047 mM were obtained for **(1), (2), (3), (5), (6), (7)** and **(8),** respectively. Chilaka et al. reported that thiourea was a good inhibitor of PPO, with low K_i value of 0.15 mM and inhibition of PPO was uncompetitive[20]. We determined that (6), (7) and (8) were a better inhibitor of PPO according to thiourea. Gulcin et al. reported that sodium diethyl dithiocarbamate was the most effective inhibitor (K_i: 1.79×10^{-6} mM) on nettle PPO[21].

Several compounds reported as PPO inhibitors were also shown to have inhibitors effect on the BPPO. The results from inhibitor studies in other plant tissues showed thiol reagents as the most effective inhibitors for those enzymes[22,23]. Reducing agents, antioxidants and enzymatic inhibitors prevent browning chemically by reducing the *o*-quinones. The effect of these reducing agents can be considered as temporary because these compounds are oxidized irreversibly by reaction with pigment intermediates, endogenous enzymes and metals such as copper. Among sulphur-containing agents, L-cysteine is an effective compound to prevent enzymic browning. Direct inhibition of PPO by cysteine through the formation of stable complexes with copper has also been proposed[24]. Halim and Montgomery showed in a series of publications that Cys can inhibit enzymic browning of pear juice concentrate more effectively than sulphite[25]. Kahn used Cys to inhibit browning of cut or pureed avocados and bananas[26]. Among the tested anti-browning reagent, the most effective ones were dithiothreitol and sodium metabisulphite[23]. The action of sulphite in the prevention of enzymatic browning can usually be explained by several processes. One is the action on *o*-quinones. The formation of quinone–sulphite complexes prevents the quinone polymerization[27]. A further action of metabisulphite on PPO is directly on the enzyme structure leading to the inactivation of PPO. Golan-Goldhirsh and Whitaker and Embs and Markakis found that during pre-incubation of PPO with sulphite (dithiothreitol, glutathione), there was a gradual loss in the ability of the enzyme to cause browning[27,28]. It has been suggested that sulphite reacts with disulphide bonds with PPO. This leads to the change in tertiary structure of enzyme and inactivation. The third process leading to PPO inhibition by bisulphate is via reduction of the intermediate quinones as described for ascorbic acid[27]. The enzyme also seemed to be sensitive to thiourea because PPO contains copper as a co-factor, the irreversible inactivation of this enzyme can be effected by substances (such as thiol compounds thiourea, -hydroxyquinoline, etc.), which remove copper from the active site of the enzyme[29]. Because sulphur is much more polarizable than oxygen, in this case, as already mentioned, a covalent bond is formed by donation of one of the S lone pairs into the empty 4s orbital of Cu. As sulphur is much "softer" than oxygen it acts as a buffer of the polarization effects caused by the metal cation, and these effects are not transmitted to the rest of the molecule in a significant amount. Hence, in this case, the conjugation of the near aminogroup is much smaller than in urea and both C–N bonds are almost equal[30].

Declaration of interest

The work was financially supported by TUBITAK (TBAG-110T133).

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chemical preparation: an efficient synthesis of N,N'-disubstituted ureas. J Org Chem 2003;68:7137–7139.
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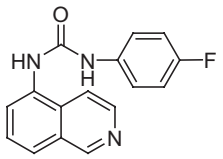
Appendix

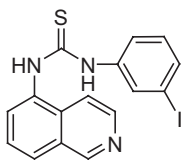
^{1}H, ^{13}C NMR and IR spectral of diarylureas

4



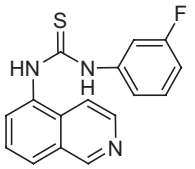
1-(isoquinolin-5-yl)-3-(4-nitrophenyl)thiourea (**3**): The experimental procedure is the same as described above. Yield 92%; mp: 264–265°C; ^{1}H NMR (DMSO-d_6): δ 3.3 (s, 3H), 7.1–7.13 (d, 1H), 7.13–7.16 (d, 1H), 7.48–7.51 (d, 1H), 7.49–7.52 (d, 1H), 7.56–7.6 (d, 1H), 7.7–7.72 (d, 1H), 7.2–7.5 (d, 1H), 8–8.02 (d, 1H), 8.48–8.51 (d, 1H), 8.8 (s, 1H), 8.9 (s, 1H), 9 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 115.93, 116.23, 118.41, 120.59 (2C), 121.46, 121.96, 124.66, 130.10 (2C), 130.93, 135.40, 136.65, 148.85, 151.01, 153.67; FT-IR ν (cm^{-1}): 3140, 2972, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH^+): 324.07.

5



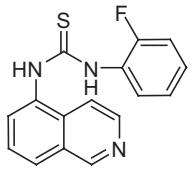
1-(4-fluorophenyl)-3-(isoquinolin-5-yl)urea (**4**): The experimental procedure is the same as described above. Yield 85%; mp: 182–183°C; ^{1}H NMR (DMSO-d_6): δ 7.55–7.57 (d, 1H), 7.58–7.61 (d, 1H), 7.75–7.78 (t, 1H), 7.81–7.89 (d, 2H), 7.92–7.99 (d, 1H), 8.19–8.22 (d, 2H), 8.34–8.37 (d, 1H), 8.92 (s, 1H), 10.3 (s, 1H), 10.5 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 122.17, 122.69, 124.99, 125.80, 126.17, 128.74, 129.79, 132.56, 135.83, 143.17, 146.94, 148.97, 151.35, 182.09; FT-IR ν (cm^{-1}): 3140, 2972, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH^+): 281.1.

6



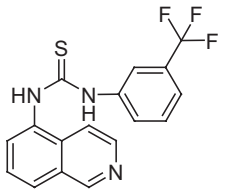
1-(3-iodophenyl)-3-(isoquinolin-5-yl)thiourea (**5**): The experimental procedure is the same as described above. Yield 89%; mp: 185–186°C; ^{1}H NMR (DMSO-d$_6$): δ 7.0–7.13 (t, 1H), 7.45–7.48 (d, 1H), 7.49–7.51 (d, 1H), 7.52–7.56 (d, 1H), 7.52–7.59 (d, 1H), 7.7–7.75 (t, 1H), 7.92–7.94 (d, 1H), 7.97 (s, 1H), 8.30–8.34 (d, 1H), 8.9 (s, 1H), 9.9 (s, 1H), 10.0 (s, 1H); ^{13}C NMR (DMSO-d$_6$): δ 94.55, 122.14, 124.13, 125.95, 126.18, 128.55, 129.81, 131.00, 132.61, 132.87, 133.75, 135.99, 141.66, 149.00, 151.28, 182.12; FT-IR *ν* (cm^{-1}): 3145, 2966, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH$^+$): 405.26.

7



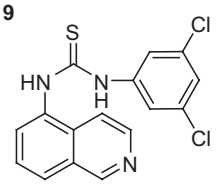
1-(3-fluorophenyl)-3-(isoquinolin-5-yl)thiourea (**6**): The experimental procedure is the same as described above. Yield 86%; mp: 186–187°C; ^{1}H NMR (DMSO-d$_6$): δ 6.8–7.0 (t, 1H), 7.2–7.3 (d, 1H), 7.34–7.4 (t, 1H), 7.5 (s, 1H), 7.51–7.53 (d, 1H), 7.51–7.54 (d, 1H), 7.78–7.81 (t, 1H), 7.95–8.0 (d, 1H), 8.34–8.39 (d, 1H), 8.9 (s, 1H), 10.0 (s, 1H), 10.01 (s, 1H); ^{13}C NMR (DMSO-d$_6$): δ 120.12, 122.11, 125.95, 126.20, 128.51, 129.77, 130.58, 130.71, 132.65, 136.08, 141.90, 142.04, 148.97, 151.27, 160.83, 182.10; FT-IR *ν* (cm^{-1}): 3140, 2966, 1595, 1541, 1498, 1444, 1332, 1263; MS(MH$^+$): 297.07.

8

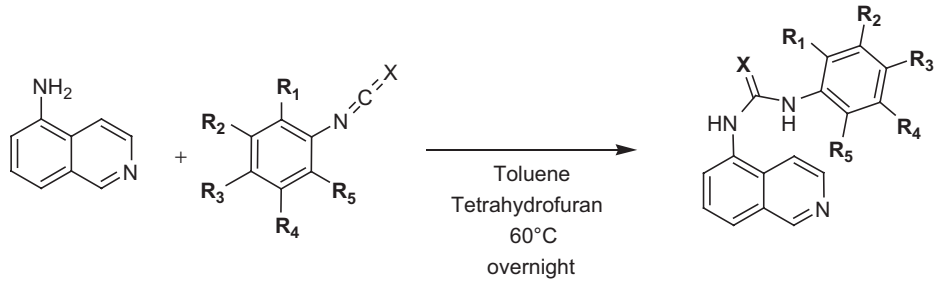


1-(2-fluorophenyl)-3-(isoquinolin-5-yl)thiourea (**7**): The experimental procedure is the same as described above. Yield 92%; mp: 184–185°C; ^{1}H NMR (DMSO-d$_6$): δ 7.1–7.2 (m, 2H), 7.2–7.26 (d, 1H), 7.5–7.6 (m, 2H), 7.56–7.59 (d, 1H), 7.73–7.78 (t, 1H), 7.95–7.98 (d, 1H), 8.25–8.37 (d, 1H), 8.9 (s, 1H), 9.5 (s, 1H), 10.09 (d, 1H); ^{13}C NMR (DMSO-d$_6$): δ 116.212, 116.479, 121.912, 124.615, 125.990, 126.173, 127.910, 128.025, 128.494, 129.292, 129.602, 132.526, 135.955, 148.940, 151.048, 182.962; FT-IR *ν* (cm^{-1}): 3140, 2972, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH$^+$): 297.07.

9



1-(isoquinolin-5-yl)-3-(3-(trifluoromethyl)phenyl)thiourea (**8**): The experimental procedure is the same as described above. Yield 96%; mp:186–187°C; ^{1}H NMR (DMSO-d$_6$): δ 7.4 (s, 1H), 7.5–7.56 (t, 1H), 7.58–7.59 (d, 1H), 7.61–7.62 (d, 1H), 7.9–8.0 (t, 1H), 8.36–8.39 (d, 1H), 8.9 (s, 1H), 10.0 (s, 1H), 10.2 (s, 1H); ^{13}C NMR (DMSO-d$_6$): δ 120.91, 121.55, 122.18, 122.951, 125.92, 126.20, 128.47, 128.59, 129.38, 129.80, 129.86, 130.13, 132.61, 135.83, 141.14, 148.96, 151.29; FT-IR ν (cm^{-1}): 3140, 2972, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH$^+$): 347.36.



1-(3,5-dichlorophenyl)-3-(isoquinolin-5-yl)thiourea (9): The experimental procedure is the same as described above. Yield 96%; mp:170–171°C; ^{1}H NMR (DMSO-d_6): δ 339 (s, 3H), 7.3 (s, 1H), 7.5–7.58 (d, 1H), 7.58–7.60 (d, 1H), 7.6 (s, 2H), 7.78–7.81 (t, 1H), 7.98–8.0 (d, 1H), 8.3–8.34 (d, 1H), 8.9 (s, 1H), 10.0 (s, 1H), 10.2 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 122.206, 122.634, 124.230, 125.883, 126.219, 128.746, 129.816, 132.584, 134.088, 135.775, 142.782, 149.001, 151.334, 182.180; FT-IR ν (cm^{-1}): 3140, 2972, 1597, 1541, 1498, 1444, 1332, 1263; MS(MH^+): 347.01.